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Differential Sensitivity to Cryopreservation of Clonogenic Progenitor Cells and Stromal Precursors from Leukemic and Normal Bone Marrow

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Abstract. The survival of human leukemic and normal progenitor cells was determined after cryopreservation. Thirteen marrows from patients with acute myeloid leukemia (AML) were studied as fresh and eight as cryopreserved samples. Marrows from five normal donors were studied as both fresh and cryopreserved samples. Although the number of bone marrow mononuclear cells (BMMC) recovered after cryopreservation was always lower than that originally stored, no significant difference was observed between the clonogenic potential of fresh and cryopreserved BMMC from either the leukemic or the normal samples. When grown in long-term bone marrow culture (LTBMC), the cultures initiated with cryopreserved BMMC failed to form a confluent stroma, and the duration of nonadherent and progenitor cell production was significantly lower than that from fresh samples. However, when these cryopreserved samples were recharged onto preformed irradiated stroma, the duration of the cultures improved significantly. We conclude that it is the bone marrow stromal cells rather than the clonogenic progenitors which are sensitive to the effects of cryopreservation. Thus cryopreservation does not appear to influence the activity of AML progenitor cells. Our results also indicate that frozen marrow can be used for LTBMC experiments if cultured on a preformed stromal layer.

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Introduction

Long-term preservation of human hemopoietic cells is possible through standardized cryopreservation techniques. Cryopreserved bone marrows are widely employed in bone marrow transplantation procedures for hematological malignancies and solid tumors [1]. Long-term storage of marrow has also been recommended for workers with high risk of marrow aplasia due to possible acute irradiation accidents [2].

The major problem associated with cryopreservation of bone marrow is the variability in recovery of viable cells, especially of hemopoietic progenitor cells [3, 4]. The repopulating ability of the cryopreserved bone marrow after ablative chemotherapy and/or total body irradiation provides evidence of survival of hemopoietic stem cells after cryopreservation [5-9].

Cryopreservation of marrow from patients in remission from leukemia has special significance. Stored marrow can be used for autologous bone marrow transplantation (ABMT) as bone marrow rescue following high-dose chemotherapy.

Different groups have reported marked sensitivity of acute myeloid leukemia (AML) progenitors to the effects of cryopreservation in rat [10] and human [11]. Cryopreservation has indeed been suggested as a means of purging the marrow of leukemic cells [11]. However, a small-scale study by *Moriyama* and colleagues [12] showed no specific loss of AML progenitors after cryopreservation.

During a study of the growth of AML bone marrow in long-term bone marrow culture

(LTBMC), we observed consistently poor growth in cultures initiated from cryopreserved bone marrow mononuclear cells (BMMC). However, the LTBMC initiated with cryopreserved leukemic marrow were particularly marked by poor stroma formation. We therefore decided to investigate whether the poor growth was due to sensitivity of leukemic cells to cryopreservation or due to the sensitivity of bone marrow stromal cells.

Materials and Methods

Patients and Samples

Bone marrow aspirates were obtained from 20 AML and myelodysplastic syndrome (MDS) patients, at the time of presentation, prior to any treatment. Marrow from one patient was also obtained after chemotherapy, during a myelodysplastic phase, making a total of 21 marrows studied. Disease type was assigned according to the FAB classification (Table I). Five marrow samples were also obtained from normal donors.

BMMC Preparation

Aspirates were diluted 1:1 in Iscove's modified Dulbecco's medium ([IMDM], GIBCO, Grand Island, NY), supplemented with 100 IU/ml penicillin-streptomycin, 10 IU/ml preservative-free heparin, and 10% fetal calf serum ([FCS], ICN-Flow, Costa Mesa, CA) and centrifuged on Ficoll-Hypaque. The BMMC obtained were washed twice in the above supplemented medium.

Table I. Patient characteristics

Patient	Sex	Age (Yrs.)	Disease	FAB type
SR	M	15	AML	M2
RP	F	57	AML	M6
SB	M	13	AML	M2
VD	F	76	AML	M2
JD	M	22	AML	M4
XP	F	30	AML	M4
ES	F	77	AML	M2
RC	M	31	AML	M1
JS	M	78	AML	M2
JM	M	54	AML	M1/M4
MK	F	40	AML	M2
MG	F	78	MDS	RARS
AC	F	33	AML	M1
GS	M	58	MDS	CMML
SK	M	60	MDS	RAEB
MB	M	40	AML	M3
JB	M	88	MDS	RA
AS	F	58	AML	M3
AA	M	67	MDS	RARS
WK	M	84	AML	M5

Cryopreservation and Thawing

Cell suspensions were mixed with FCS (final concentration 45% v/v), IMDM (final concentration 45% v/v) and dimethyl sulfoxide ([DMSO]; final concentration 10% v/v) and rapidly placed on dry ice for 4 h prior to liquid nitrogen storage. Frozen cells were thawed at 37°C and washed twice.

Committed Bone Marrow Progenitor Assay

Committed bone marrow progenitor cells were assayed using a modification of the method described by *Fausner* and *Messner* [13, 14]. Briefly, BMMC were suspended at 10^5/ml in IMDM supplemented with 0.9% methylcellulose (Terry Fox Laboratory, Vancouver, British Columbia, Canada), 30% FCS (ICN-Flow), 200 mM glutamine, 1% deionized bovine serum albumin (BSA) and 10^{-4} M 2-mercaptoethanol. Duplicate 35 mm dishes were set up with 1 ml each of the cell suspension. Each dish was inoculated with recombinant human interleukin 3 ([rIL-3]; 50 ng/ml; Sandoz Pharma, Basel, Switzerland), recombinant human granulocyte-macrophage colony stimulating factor ([rGM-CSF]; 50 ng/ml; Sandoz Pharma), recombinant human erythropoietin ([rEpo]; 2 U/ml; CILAG Ltd., High Wycombe, UK) and 5% conditioned medium from the human bladder carcinoma cell line, 5637. Dishes were incubated at 37°C in 5% CO_2/95% air. On day 14, granulocyte-macrophage colony forming units (CFU-GM), erythroid burst forming units (BFU-E) and mixed colonies (CFU-GEMM) consisting of granulocyte-macrophage and erythroid elements were scored. Individual colonies were harvested and stored in 100 µl sterile water at –70°C. Identification of some colonies was confirmed by cytospin preparations using Wright-Giemsa stain.

LTBMC

A modification of previously established techniques was used for the establishment and maintenance of LTBMC [15, 16]. Briefly, BMMC were seeded into 25 cm^2 tissue culture flasks at 10×10^6 (1° cultures) or at 5×10^6 on

preformed irradiated stroma (2° cultures) in 10 ml of Fischer's medium (GIBCO) supplemented with 10% FCS, 10% horse serum, 100 IU/ml penicillin-streptomycin and 10^{-6} M hydrocortisone sodium succinate. Flasks were incubated at 33°C in 5% CO_2/95% air. At weekly intervals, cultures were demi-depopulated and fed with an equal volume of fresh supplemented Fischer's medium. The nonadherent cells were counted, resuspended in IMDM and assayed for committed progenitor cells. Cultures were considered to have terminated when no nonadherent cells or no clonogenic cells were obtained in at least two successive weeks.

Statistical Analysis

Results are expressed as mean ± SE [no. of observations]. Groups were compared by the *t*-test or paired *t*-test as appropriate.

Results

Total Nucleated Cell Recovery

Defined numbers of leukemic and normal BMMC were cryopreserved as described in the Materials and Methods section. The number of BMMC recovered from the cryopreserved samples after thawing was always considerably less than that originally stored. However, no significant difference in the effect of cryopreservation was observed between leukemic and normal samples (leukemic mean 41%, normal mean 47%).

Clonogenic Cell Recovery

When defined numbers of viable BMMC from cryopreserved AML samples were plated in clonogenic assays, significant growth was seen in most cases. When a series of assays from fresh AML BMMC was compared with the series from frozen AML BMMC, the differences were not significant (fresh 78 ± 29 colonies/10^5 cells [13]; frozen 155 ± 75 colonies/10^5 cells [7]; $p = 0.27$). This indicates that, while there were losses of total BMMC, there was no preferential loss of clonogenic cells.

Similarly, the clonogenic potential of normal cryopreserved BMMC samples after freezing and thawing was not significantly different from that of the same samples grown from fresh BMMC (fresh 161 ± 40 colonies/10^5 cells [4]; frozen 171 ± 55 colonies/10^5 cells [4]; $p = 0.87$).

Nonadherent (NA) Cell Recovery from LTBMC

Primary cultures initiated from cryopreserved leukemic BMMC produced NA cells for a shorter period on average than cultures from the fresh leukemic cells, but the difference was not statistically significant (fresh 5.2 ± 0.5 weeks [13]; frozen 3.9 ± 0.64 weeks [8]; $p = 0.12$). With normal BMMC, a significant difference was observed between fresh and cryopreserved samples (fresh 4.8 ±0.37 weeks [5]; frozen 3.4 ± 0.4 [5]; $p = 0.005$).

Clonogenic Cell Recovery from LTBMC

On comparison of the duration of clonogenic potential in 1° LTBMC from fresh and frozen leukemic BMMC, a much more dramatic difference was seen. Colony growth did not last beyond two weeks in frozen samples compared with up to nine weeks in fresh samples (fresh 3.7 ± 0.56 weeks [13]; frozen 1.4 ± 0.18 [8]; $p = 0.007$) (Tables II and III).

As with the leukemic BMMC, a marked difference in duration of clonogenic potential of fresh and cryopreserved normal BMMC was observed (fresh 4.2 ± 0.66 weeks [5]; frozen 2.8 ± 0.45 weeks [5]; $p = 0.05$) (Table IV).

Stroma Formation in LTBMC

Fresh BMMC from leukemic or normal bone marrow consistently formed a confluent stroma within two weeks of culture. However,

Table II. Clonogenic potential of NA cells recovered from 1° LTBMC of cryopreserved AML/MDS samples

	Colonies/Flask				
Pts.	W0	W1	W2	W3	W4
SR	0	0	2	0	
RP	36,500	31	0	0	0
SB	430	123	+++	0	0
VD	9,400	592	0	0	0
JD	8,300	42	0	0	0
XP	210	173	2	0	0
ES	50,420	961			
WK	0	3,540	0	0	0

Mean duration 1.4 ± 0.18 weeks

+++ = a large number of clusters seen, but not counted. At the end of the cultures, 0 indicates that nonadherent cells were cultured but no colonies were obtained. A blank indicates that there were no nonadherent cells produced.

Table III. Clonogenic potential of NA cells recovered from 1° LTBMC of fresh AML/MDS BMMC

	Colonies/flask									
Pts.	W0	W1	W2	W3	W4	W5	W6	W7	W8	W9
JS	450	14	8	0	0					
JM	7,500	3,400	2,272	12	5	6				
MK	0	0	0	0	8	6				
JS (MDS)	1,400	160	0	0	0	0				
AS	ND	1,640	1	4	0					
MG	9,500	3,350	620	93	0					
AC	0	2	12	6						
GS	1,400	0	18	12	1	2				
SK	24,400	436	107	98	0	0				
MB	35,147	13,804	6,462	0	0					
JB	160	3	17	2						
AA	670	630	7	1	0					
RC	8,400	ND	541	5	24	80	20	3	ND	8

Mean duration 3.7 ± 0.56 weeks.

ND = not determined

frozen BMMC from either leukemic or normal bone marrow failed to form a confluent stroma even after four to five weeks. The absence of a confluent stroma may be sufficient in itself to explain the poor growth of these cultures. We therefore investigated whether cryopreserved BMMC would maintain long-term hemopoiesis on a preformed, irradiated stromal layer.

2° LTBMC on Preformed Stroma

Cryopreserved leukemic BMMC were used to establish primary LTBMC or were used to recharge preformed irradiated stromal layers established from normal donors (2° LTBMC). The duration of the total nucleated cell recovery was not significantly different in 2° LTBMC from that in 1° cultures (1° 4.2 ± 0.8 weeks [5]; 2° 4.6

Table IV. Clonogenic potential of NA cells from fresh and cryopreserved 1° LTBMC BMMC of normal donors

	Colonies/flask						
	W0	W1	W2	W3	W4	W5	W6
FRESH							
Donor							
M	10,000	615	143	0	0	0	
G	31,000	336	210	1,118	50	0	
Mu	17,000	458	182	48	196	2	
H	8,000	360	244	21	59		
J	ND	1,136	140	64	3	6	1
Mean duration 4.2 ± 0.66 weeks							
FROZEN							
Donor							
M	8,300	2,662	13				
G	17,000	359	206	21	0		
Mu	20,000	488	287	98	0		
H	9,000	213	31,142				
J	22,000	1,116	73	10	0		
Mean duration 2.8 ± 0.45 weeks							

ND = not determined

± 0.9 weeks [5]; $p = 0.58$). However, the duration of recovery of clonogenic cells was significantly greater in 2° cultures (1° mean 1.4 ± 0.18 weeks [8]; 2° 2.2 ± 0.2 weeks [5]; $p = 0.013$) (Table V) and was not significantly different from that of fresh 1° cultures ($p = 0.15$). Fresh leukemic BMMC were not cultured on preformed stroma.

In similar experiments with normal fresh marrow, 1° and 2° LTBMC did not show a significant difference in the duration of total nucleated cell recovery or in duration of clonogenic potential (1° 4.2 ± 0.66 weeks [5]; 2° 3.8 ± 0.37 weeks [5]; $p = 0.37$). However, the cryopreserved samples showed a marked increase after recharging, both in duration of NA cell production (1° 3.4 ± 0.4 weeks [5]; 2° 5.0 ± 0.0 weeks [5]; $p = 0.016$), and in duration of clonogenic potential (1° 2.8 ± 0.2 weeks [5]; 2° 4.6 ± 0.24 weeks [5]; $p = 0.0008$) (Table VI). The duration was not significantly different from that of fresh 1° cultures ($p = 0.88$).

Discussion

Although in this study we found a significant loss of mononuclear cells (MNC) after cryopreservation, we did not find a specific loss of either normal or leukemic progenitor cells. Since fresh and cryopreserved samples from the same patient were not studied, we cannot rule out some quantitative decrease in individual cases. It must be noted that both colony growth and growth factor responsiveness vary widely among individual cases of AML (see *Lemoli et al.* [17]). Nevertheless it is striking that the mean colony number from the cryopreserved samples was over twice that of the fresh controls, and this is not consistent with a generalized sensitivity of leukemic progenitors to cryopreservation. Within our culture system, most leukemic progenitors give rise to colonies containing differentiated cells. However, we have demonstrated the presence of *ras* gene mutations in a significant subset of such colonies, including many in the present experiments, showing that they must derive from the leukemic clone (*Zaheer et al.*, manuscript in preparation).

Interest in cryopreservation as a means of leukemic bone marrow purging was stimulated by the dramatic loss of rat leukemic cells demonstrated by *Hagenbeek* and *Martens* [10]. *Moriyama et al.* [12] studied four AML patients for recovery of leukemic progenitors after freezing. Leukemic progenitors were recovered after cryopreservation in three of the four cases; however, although there was an apparent trend to decreased progenitor numbers after freezing, this was not statistically significant. *Allieri et al.* [11] studied five leukemic patients with six variations on the typical freezing protocol. There was no statistically significant difference in results from the different freezing methods, but there was a consistently lower (by about 20%) recovery of AML CFU compared with normal CFU.

Our results appear to be in clear contradiction to those of *Allieri et al.* The freezing method we employed was relatively harsh, because controlled rate freezing was not used, but is comparable to methods employed by *Allieri et al.* Our cell culture methods, in contrast, were distinctly different, since *Allieri et al.* used an irradiated peripheral blood leukocyte feeder layer to support clonogenic growth, while we used recombinant growth factors. It is at least conceivable that particular types of cell damage might be accentuated or compensated for by different culture conditions. One other

Table V. Clonogenic potential of NA cells recovered from 2° LTBMC of cryopreserved AML/MDS samples

	Colonies/Flask					
	W0	W1	W2	W3	W4	W5
Pts.						
SR	0	0	10	0	0	0
RP	18,250	11	0	638	0	
SB	215	11	+++	0	0	
VD	4,750	50	29	0	0	
JD	4,150	25	17	0	0	0

Mean duration 2.2 ± 0.2 weeks

+++ = a large number of clusters observed but not counted

Table VI. Clonogenic potential of NA cells recovered from 1° and 2° LTBMC from cryopreserved BMMC of normal donors

	Colonies/Flask					
	W0	W1	W2	W3	W4	W5
1°						
Donor						
J	22,000	1,116	73	10	0	
M	8,300	2,662	13			
G	17,000	359	206	21	0	
Mu	20,000	488	287	98	0	
H	9,000	213	311	42		
Mean duration 2.8 ± 0.2 weeks						
2°						
Donor						
J	27,500	1,620	313	178	660	6
M	8,300	430	160	156	69	0
G	8,500	180	304	96	34	0
Mu	10,000	462	489	85	77	16
H	4,500	288	272	90	135	44
Mean duration 4.6 ± 0.24 weeks						

significant difference between our experiments is that *Allieri et al.* used peripheral blood samples in three of their cases, while all our work was done on bone marrow.

It is conceivable that different subsets of AMLs may differ in their sensitivity to cryopreservation. Our results would tend to obscure such an effect, since we did not study paired samples. In this context it may be significant that all of *Allieri et al.*'s results were on only two FAB subtypes, M2 and M4, while we studied representatives of a greater variety of FAB subtypes.

When LTBMC were initiated with either normal or leukemic cryopreserved BMMC, we observed a consistent failure to form a confluent stromal layer and a foreshortened period of in vitro hemopoiesis. Although there was significant loss of MNC with cryopreservation, no specific deficit of progenitor cells could be detected, and all cultures were initiated with equal numbers of viable BMMC. We therefore concluded that the failure of the LTBMC was due to the absence of a functional stromal layer. Hemopoiesis early in LTBMC is largely maintained by the same progenitors that will support clonogenic assays. However, late in LTBMC hemopoiesis is maintained by a distinct cell type, the long-term culture initiating cell (LTCIC) [18], which may include true multipotent stem cells. We could not therefore exclude a specific loss of LTCIC on cryopreservation, so, in order to investigate this 2° LTBMC were initiated by adding BMMC to preformed irradiated LTBMC stromal layers. With fresh normal BMMC there was no significant difference between 1° or 2° culture. When frozen normal BMMC were grown in 2° cultures, the duration of clonogenic cell production was increased to a level not significantly different from that of fresh 1° cultures. Thus it seems that the failure of clonogenic cell production in LTBMC from normal cryopreserved BMMC can be attributed to the effect of freezing on stromal cells and can be restored by use of a preformed stromal layer.

Similarly, when cryopreserved leukemic BMMC were cultured on a preformed irradiated stromal layer, the duration of hemopoiesis was increased relative to primary cultures and was not significantly different from that of fresh AML cells grown in primary culture.

Our results show that it is possible to use cryopreserved BMMC from both normal and AML subjects for LTBMC, as long as a preformed stromal layer is used. They also indicate that cryopreserved AML bone marrow cannot be assumed to have been purged of leukemic progenitors. It is still possible that particular aspects of cryopreservation might be purgative, or that particular

subtypes of leukemia might be sensitive to cryopreservation, and we would suggest, based on our own results, that the influence of culture conditions on survival should be taken into account.

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